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AN INVESTIGATION OF THE MEMORY RESPONSE OF THE LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS

ANNUAL REPORT

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In the present studies we have learned how to enhance the initial mucosal immune response of the intestine to the enteropathogen Shigella flexneri. By administering heat-killed Shigella X16 a day prior to the oral challenge with live Shigella X16, a significant enhancement of the early secretory IgA response to Shigella lipopolysaccharide was demonstrated. This response was not dependent on the use of an adjuvant. Further, the mucosal immune response does not require priming by pathogenic strains. We have begun comparing the uptake of nonpathogenic with pathogenic strains of shigella and determined that both are readily sampled by the specialized epithelium which overlies Peyer's patches in the gut. This may serve as the site of entry for the pathogenic strains. The other major epithelial cell type which has been implicated in bacterial sampling is the Paneth cell. In the present studies, we show conclusively that the numbers of these cells in the epithelium are directly related to bacterial flora in the gut lumen. In addition to the intact bacteria, we have begun studies examining the mucosal immune response to Shiga toxin. Preliminary data are presented which demonstrate a dose dependent inhibition of the toxin effects of Shiga toxin

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19. ABSTRACT (Continued)

by specific secretory IgA. As data on the mucosal immune response to shigella antigens accumulates, it is becoming clear that manipulation of this response can be used to achieve a protection against these and other mucosal infections.

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Summary

Mucosal immunity to some enteropathogens occurs naturally following infection. By learning how to control the initiation of the mucosal immune response, it is possible to develop vaccines against a wide variety of enteropathogens and their toxic products. In the past year, our investigations have centered around enhancing the initial mucosal immune response to Shigella flexneri. To this end, we have used a combination of parenteral antigen administration using heat-killed Shigella and oral administration of a live Shigella vaccine strain to augment the initial mucosal immune response to this pathogen. We have found that this augmentation is not dependent on the use of an adjuvant. Therefore, it would be possible to use such augmentation in a human population. Further, we have looked at the mucosal immune response to Shiga toxin. We have found that a significant IgA anti-Shiga toxin response is present in intestinal secretions following direct intraintestinal immunization with this antigen. In collaborative work with Dr. J. Edward Brown, we found that the IgA anti-Shiga toxin titer correlated precisely with anti-Shiga toxin protective activity in secretions. This exciting information indicates that mucosal vaccines against Shigella should be able to confer protection. To better understand the cellular events, and thereby, provide a logical method for determining vaccination sequence, we have begun studies to follow the cellular events initiated by our mucosal vaccines. In the present report, we described the IgA anti-Shigella activity present at a distal site (spleen) following the combined parenteral and oral immunization regimen which augmented the primary IgA response described above. Another important factor in mucosal immunity vaccination involves the ability of antigens to be taken up by the antigen processing cells, M cells, overlying Peyer's patches. In the present studies, we have found that uptake of Shigella flexneri does not correlate with mucosal immune response as much as it correlates with the inherent pathogenicity of the microorganism. Pathogenic strains of Shigella were taken up much more readily by the dome epithelium (enriched in M cells) than were nonpathogenic strains. This implies that the M cell may serve both as initial site of antigen processing and as a portal of entry for enterpathogens. Lastly, we have studied another cell type which may be involved in mucosal immune defense. The Paneth cell has been poorly understood with regard to its in vivo function. In the present studies, we demonstrate that the hyperplasia of Paneth cells which occurs in our chronically isolated ileal loop system in rabbits is directly related to the flora of the gut lumen. Since Paneth cells are known to be phagocytic, it is probable that these cells are responding to the presence of excessive numbers of microorganisms in the gut lumen. While the exact functions of these cells are still unclear, our future studies will attempt to purify this cell population and determine their ability to phagocytose and destroy enteropathogens which are coated with antigen specific IgA or IgG.

Foreword

In conducting the research described in this report, the investigators followed the "Guic or Care and Use of Laboratory Animals" prepared by the The Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (NIH Publication 85-23 Revised 1985).

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Introduction

In the past decade, several model systems have been developed to study the details of the production of secretory IgA by the mucosal surface. Until recently, most attention in vaccine research has been directed toward establishing humoral (predominantly IgG) responses to the infectious agents. With the recognition, however, that secretory IgA is overwhelmingly the predominant immunoglobulin along mucosal surfaces and likely responsible for host defense, much work has been directed at understanding initiation of the secretory IgA response and at establishing a secretory IgA memory response. Also, as described later in this report, the mechanisms by which secretory IgA effects protection are being sought.

In our laboratory, we have been able to follow the secretory IgA responses sequentially by using a chronically isolated ileal loop model in rabbits as a probe (1). With this Thiry-Vella loop model system to provide secretions, we have characterized the intestinal IgA responses to cholera toxin, Shigella flexneri, Salmonella typhi, and, most recently, Shiga toxin, (2-6). Our use of these Thiry-Vella loops to probe mucosal immunity is a logical extension of the present understanding of antigen processing and lymphocyte trafficking following antigen administration.

Following oral administration of antigen, specialized surface epithelial cells which overlie lymphoid follicles, Peyer's patches, and the appendix have been identified which take up both luminal macromolecules and microorganisms for antigen processing. These "M" cells can take up macromolecules such as horseradish peroxidase within a few minutes of their application to the intestinal lumen (7,8). Our previous studies found that isolated follicles also contain these cells (9). In the present report, we will review the uptake of Shigella flexneri by these cells and the role this plays in both antigen processing as well as in the pathogenicity of this microorganism. Following uptake of antigen, the material is brought to the underlying lymphoid tissues which are enriched in precursor B lymphocytes for an IgA response. At this stage of development, most B lymphocytes in the gut-associated lymphoid tissues (GALT) express surface IgM and/or IgD. Immunoregulatory T lymphocytes are present in GALT which influence the development of IgA precursor B lymphocytes (10,11). These regulatory T cells are only beginning to be understood in relationship to their potential to influence the result of mucosal vaccination. Populations currently understood include the switch T lymphocytes described by Kawanishi et al. (10). Under the influence of lipopolysaccharide, clones of these switch T cells alter the surface immunoglobulin expression of Peyers patch B lymphocytes from IgM to IgA. Determining the sequence, location and frequency with which this type of switch occurs after oral vaccination could be important in orchestrating an IgA response to enteropathogens. Other laboratories have described helper T cells for the IgA response. These helper cells encourage differentiation of B lymphocytes which already bear surface IgA toward becoming mature plasma cells (11). In addition to regulatory T cells, it is clear that the B lymphocytes within GALT have an inherent genetic propensity for becoming IgA synthesizing and secreting plasma cells (12). By studying the in vitro capability of lymphocytes from several pertinent

sites (GALT, lamina propria cells, spleen, peripheral lymph nodes, mesenteric lymph nodes), we will learn the location of cells precommited to form IgA against enteropathogens following mucosal priming with antigen. This will result in a more logical approach to developing vaccines against a wide variety of enteropathogenic agents.

In our previous work, we documented several major features of the secretory IgA response that have relevance to potential vaccine programs. First, an IgA response to S. flexneri is elicited best by an oral immunization (3). Second, a local secretory IgA memory response can be elicited by oral immunization with live but not killed S. flexneri (5). Lastly, while the vaccine needs to be live, it need not be pathogenic. Strains of noninvasive Shigella and others which lack the 140 megadalton virulence plasmid were equally effective to virulent strains of Shigella in eliciting a mucosal memory response (6).

In the present studies, we have concentrated on the role of antigen uptake by the intestine in establishing the mucosal immune response to shigella. By carefully orchestrating a combined parenteral and oral immunization regimen with S. flexneri, we have been able to enhance the primary secretory IgA response to S. flexneri LPS. We have looked, for the first time, at the mucosal immune response to Shiga toxin, and have demonstrated a profoundly effective protection by secretory IgA. Preliminary studies indicate that the uptake of virulent microorganisms by the follicle-associated epithelium which may be taken up by M cells as the portal of entry (13). This could have important implications both for traditional enteropathogens and newer agents such as the human immunodeficiency virus (HIV) may serve as the site for invasion. Lastly, we have reviewed the influence of the intestinal flora on the poorly understood Paneth cell. We established that this cell undergoes dramatic response to the microbial content of the gut lumen. This raises important questions about to role of these cells in either antigen processing (as they are known to be phagocytic) or in host defense against enteropathogens (as Paneth cells are known to contain lysozyme) (14).

Methods

Preparation of Chronically Isolated Ileal Loops. The surgical creation of chronically isolated ileal loops in rabbits has been described in detail previously (1). Briefly, 3 kg New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. Twenty centimeters of ileum containing a Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunnelled subcutaneously to the nape of the neck where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis. The midline incision is closed in two layers.

Each day, about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid and mucus expelled from the tubing is studied for specific immunoglobulin content. A subsequent flush with 20 ml of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes of air. With proper daily care, > 90% of our rabbits have completed experiments lasting 2 months.

Enzyme-linked Immunosorbent Assay (ELISA). Microtiter wells are coated with a solution containing S. flexneri lipopolysaccharide (LPS) (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the LPS antigen solution is removed and the wells are washed with a phosphate-buffered saline solution (PBS) containing 0.05% Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated and uncoated wells (the latter to control for nonspecific adsorption) for 4 hours. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA or sheep anti-rabbit IgG (both are isotype specific affinity column purified in our laboratory using methods previously described (15)). After an incubation of 4 hours, the wells are again washed with PT and the substrate reaction is carried out with p-nitrophenyl phosphate in carbonate buffer pH 9.8. The kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. The OD 405 mn (read on a Titertek Microelisa Reader) of the uncoated wells is subtracted from that of the coated wells. Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (16).

The data are analyzed using the RS1 software system. Data are presented as geometric means, as others have noted that this better reflects the logarithmic kinetics of the local immune response after immunization (17). For each day's result, the variance is expressed together with the mean.

Antigen Preparations Used. Four antigen preparations were employed in the present studies: 1) Shigella flexneri M4243 (which can invade intestinal mucosa and persists in the epithelium), 2) Shigella X16 (a hybrid of S. flexneri and E. coli-which invades the intestinal mucosa but does

not persist within the epithelium, 3) S. flexneri M2457-0 (which does not invade, but which possess the 140 megadalton virulence plasmid), 4) S. flexneri M4243A$_1$ (which lacks the virulence plasmid and shows no invasiveness). All strains are tested for invasion using the Sereny test. The Sereny test is performed weekly on strains to assure the invasive, or noninvasive activities for Shigella uptake studies.

In Vivo Assay for Uptake of Shigella flexneri by Follicle-Associated Epithelium and Villi. To determine the relationship between the virulence of the microorganism and its uptake by the isolated follicle epithelium, an in vivo assay procedure was employed. 10 cm isolated ileal loops were created in conventional New Zealand bred rabbits. A single dose containing 10^8 Shigella flexneri was injected into this acute group. At 30, 90, and 180 minutes, these loops were removed and frozen sections were prepared. These sections were fixed in methanol and stained with Giemsa. For each time, at least 10 sections of Peyer's patch and adjacent villi were examined for attachment and uptake of the Shigella flexneri. Histologically, these sections were divided into three areas: 1. the follicle associated epithelium overlying the dome areas in Peyers patches (known to be enriched in "M" cells). 2. the villi between the dome areas within the Peyer's patch. 3. villi which were outside of the Peyers patch area. Evaluation was performed using oil emersion light microscopy. Since the normal flora of the rabbits ileum contains less that 10^4 microorganisms, for statistical purposes, less than .1% of the flora visualized were from other microorganisms. Further, the Shigella flexneri have a characteristic size and shape which, under the circumstances of this study, were readily recognizable using this technique. Electron microscopy was performed on some sections demonstrating the characteristic rod-shaped structure and the typical "M" cell location. Results were expressed as microorganisms per dome area or microorganisms per villus.

Mucosal Immune Response to Shiga Toxin. Two groups of rabbits were used in this study: Two normal rabbits, three specific pathogen-free rabbits. Following creation of a chronically isolated ileal loop in each rabbit, three weekly intraloop doses of Shiga toxin was administered (the first dose being given on the day of surgery-antigen day 0). The Shiga toxin preparation used for this study was a post-DEAE fraction, provided by Dr. Ed. Brown of the Walter Reed Army Institute of Research. Dr. Brown's laboratory found that this preparation contained 10^7 units of toxin per ml of fluid. Each loop dose consisted of 0.5 ml of Shiga toxin fraction plus 3.5 ml of saline. As in our other studies, the ileal loop was flushed daily of mucus and secretion. The 4 ml of toxin preparation were then injected into the loop, followed by 2 ml of air. One of the tubes from the loop was immediately taped to keep the toxin from spilling out of the loop. Ileal loop secretions from each animal were collected daily for 1 month and assayed for specific IgA against Shiga toxin.

Samples of the flushes from the first two rabbits for several antigen days were sent to Dr. Brown who tested them for their anti-Shiga toxin activity using his in vitro assay.

Mononuclear Cell Isolation. At time of sacrifice, rabbits from various immunization groups had peripheral blood, Peyer's patches, mesenteric lymph nodes, spleen and axillary lymph nodes removed under asceptic con-

ditions. For the peripheral blood, the buffy coat was placed on lymphocyte separation medium and centrifuged at 400 x g at room temperature for 30 minutes. The cells at the interface were removed, characterized and used as mononuclear cell preparations. Tissues were cut into 1 cm square fragments with a sterile blade and placed on sterile wire mesh. The cells were carefully teased apart and passed through the mesh. This material was centrifuged at 400 x g at room temperature for 7 minutes. The pellet was gently resuspended and washed twice in RPMI 1640. The total cells and viability were determined. A Wright stain preparation was examined to determine the differential of the isolated cells.

Mononuclear Cell Phenotypes. Cell surface immunoglobulin positive and phagocytic cells were assayed together. 10^6 viable mononuclear cells were suspended in 1 ml of a 1.1 micron latex bead/complete media suspension. This was incubated at 37°C in a humidified 95% air and 5% CO2 incubator from 1 to 2 hours. The cells were then washed in a cold 4°C phosphate buffer of pH 7.4 in 0.1% sodium azide. Thereafter, it was incubated with 100 microliters of a 1:20 dilution of fluorescein-labeled goat anti-rabbit IgA or anit-rabbit IgM (Cappel) in an ice bath for 30 minutes. The cells were then washed three times in 2 ml of cold buffer and resuspended in 1 drop of glycerol:PBS 9:1. The number of surface immunoglobulin positive (sIg) and phagocytic cells (at least 3 latex beads per cell) were enumerated using a Leitz microscope having both epifluorescence and phase contrast capabilities. At least 100 cells were counted for each calculation. Cytoplasmic immunoglobulin-containing cells were assessed using ethanol-fixed cytospin preparations, these were assayed with fluorescein-labeled anti-polyvalent rabbit immunoglobulins (Cappel). The cytoprep was then washed 3 times with standard buffer, mounted with glycerol:PBS and at least 100 cells were counted.

In Vitro Mononuclear Cell Cultures. 10^5 mononuclear cells were added to each row of a 96 well, polystyrene microtest III tissue culture plate (Becton Dickenson). The wells are flat-bottomed. Cultures were placed in a humidified, 95%/5% CO2 37°C incubator. At the times indicated in the result section, 3 wells for each tissue were aspirated. Cellular debris was removed by centrifugation at 440 x g for 15 minutes and the supernatants were stored at -20°C until assayed. Assays were performed using the above described ELISA technique.

Paneth Cell Studies. In each of 15 New Zealand white rabbits isolated ileal loops were created. One group of rabbits had the loop flushed daily per routine. Loop fluids and normal ileum were cultured to determine degree of bacterial colonization In the second group of rabbits, an antibody solution containing 10 mg/ml neomycin sulfate and 2.5 mg/ml bacitracin in sterile saline together with 50 ug/ml of gentamicin were added to the isolated ileal loops. The nonabsorbable nature of these 3 antibiotics was a major requirement for their selection over other more commonly used agents. Animals were sacrificed at 2 weeks after surgery to determine the effect of the antibiotics on the flora and on the Paneth cell structure.

Coded Histologic Study Utilized Routine Hematoxylin and Eosin Stained Section. One section was examined for each tissue specimen. Paneth cell hyperplasia was estimated using an ocular micrometer with the measurement

of the height of the Paneth cell column (extension from crypt base into higher levels of the crypt) in 20 consecutive well-oriented crypts of each section. Crypt depth and villus height was similarly estimated by ocular micrometer. With 12 consecutive well-oriented crypt-villus pairs measured in each section. Average values were used to represent each parameter for each animal. Lamina propria heterophiles and epithelium mitotic figures were counted in 10 high power fields (400x) of the crypt region. Goblet cells and intraepithelial lymphocytes (IEL) were counted per 500 epithelial nuclei on the sides of well-oriented villi.

Results

I. Combined Oral and Parenteral Immunization to Achieve an Enhanced Primary Mucosal Immune Response to Shigella Flexneri.

While our earlier studies have shown the existence of a secretory IgA memory response to Shigella flexneri, elicitation of this vigorous mucosal immunity has required three weekly oral priming doses with live Shigella. In the present studies, we sought information about the potential to arm the secretory IgA system more rapidly by combining parenteral with oral immunization routes.

A. Immunogenicity of heat-killed Shigella. Previous studies in our laboratory demonstrated that heat-killed Shigella have been ineffective in simulating a mucosal memory response (5,6). To determine whether the heat-killed preparations can be effective stimulants for mucosal immune response, or whether they have lost key antigenic determinants, a single dose of 10^{10} heat-killed Shigella X16 was injected directly into each of 5 Peyer's patches (0.2 ml/ Peyer's patch) at the time of surgical creation of the isolated ileal loop in these animals (Table I). The 7 rabbits in this group developed significant increases in the secretory IgA anti-Shigella

Table I. Immunization Schedule

Group	Antigen[1]	Route	Day
I	Heat-killed Shigella X16	Peyer's Patch[2]	0
II	Live Shigella X16	Oral[3]	0
III	Heat-killed Shigella X16	Intramusclar[4]	-1
	Live Shigella X16	Oral[3]	0
IV	Heat-killed Shigella X16 in CFA	Intramuscular[4]	-1
	Live Shigella X16	Oral[3]	0
V	Heat-killed Shigella X16 in CFA	Intramuscular[4]	-1

1. All doses contained 10^{10} shigella.
2. Antigen injected into each of 5 Peyer's patches including the one in the isolated ileal loop.
3. Oral dosage given under mild anesthesia via neasogastric cannula.
4. Intramuscular dosage given with CFA.

LPS activity in their loop secretions over day 0 value by the fourth day after surgery (Figure 1). This is significantly sooner than responses which were achieved when antigen preparations are given orally (see later Group II). In a few secretions, weak IgG activity to Shigella LPS was detected (data not shown). In the serum, the opposite specific antibody activity was seen. As shown in Table II, the IgG anti-Shigella LPS activity rose to a geometric mean of 1.038 and did not significantly decline by the end of this study at one month. In contrast, the serum IgA activity to Shigella LPS was weak throughout the study (Table II). These findings indicate that the heat-killed Shigella preparation was immunogenic for both the local IgA and systemic IgG response following immunization directly into Peyer's patches. Therefore, antigenic determinants requisite for stimulating mucosal immunity are present in these preparations. This information was important for allowing the subsequent studies in this series of experiments.

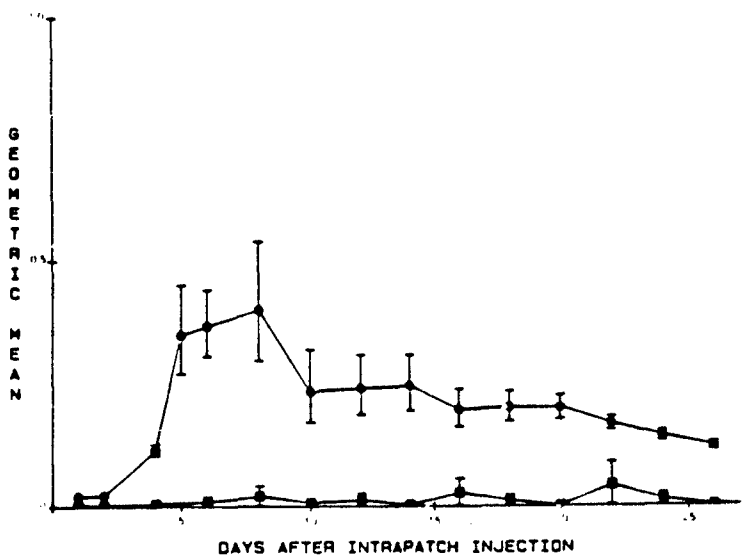

Figure 1. This depicts the Geometric mean IgA anti-shigella LPS (circles) and IgG anti-shigella LPS (squares) responses in isolated ileal loop secretions from rabbits given a single injection of heat-killed shigella into their Peyer's patches on day 0. Standard errors of the means (SEM) are indicated.

Table II. Serum IgG and IgA Activity to Shigella LPS from Rabbits Given Antigen Directly into Peyer's Patches.

Days Post-Immunization[1]	IgA	anti-LPS[2]	IgG	anti-LPS[2]
6-7	.263	(.228-.305)	.479	(.363-.630)
8-14	.268	(.239-.299)	1.038	(.922-1.165)
15-21	.206	(.199-.213)	.927	(.814-1.058)
22-28	.192	(.184-.200)	.824	(.570-1.193)

1. Data not available for preimmunization. For comparison, Tables III and IV show values of .018 and .017 for IgA and .015 and .026 as Geometric means of unimmunized rabbits.
2. Data expressed as Geometric means with variance as described in the Methods section.

B. Immune responses following oral stimulation with live Shigella X16. Group II (Table I) was a repeat of an experiment performed approximately 8 years ago in this laboratory. This control group of rabbits received a single oral dose of 10^{10} live Shigella X16. The mucosal immune response from these animals was a typical local primary IgA response (Figure 2) compared to the results from animals examined 8 years ago, there was no significant difference at any point. The reproducibility of this model system in an outbred species was excellent. The first significant increase in the IgA anti-Shigella activity over preimmunization values is found on day 6 with the response peaking on day 8. As in our previous studies, no IgG anti-Shigella LPS activity was found in the intestinal secretions (Figure 3). Further, in the serum, no IgG or IgA activity to Shigella was detected in the serum. These findings indicate that a single live oral dose of Shigella X16 elicits predominantly a secretory IgA response. Based on these, and the previous findings (group I) we determined that combining parenteral immunization with heat-killed Shigella and oral immunization could result in an enhanced primary IgA response.

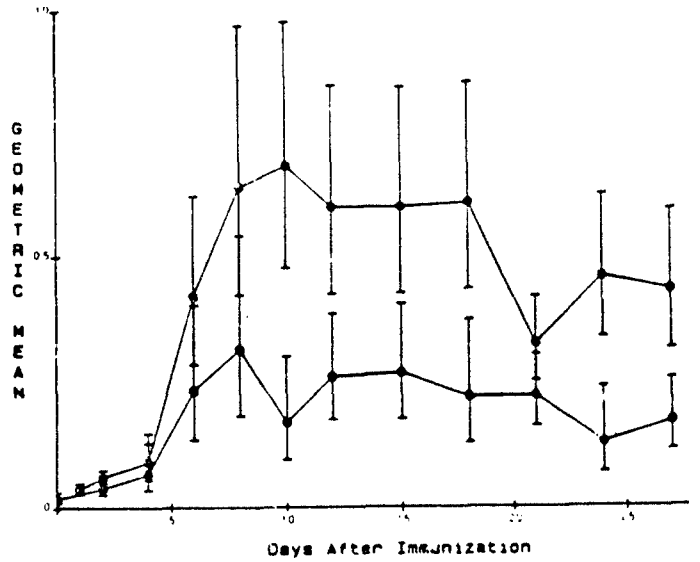

Figure 2. Geometric mean IgA anti-shigella LPS responses in isolated ileal loop secretions from rabbits given a single oral dose of live shigella on day 0 (closed circles) and from rabbits given a combined parenteral dose of antigen on day -1 with the single oral dose on day 0 (open circles) are shown. SEM are indicated.

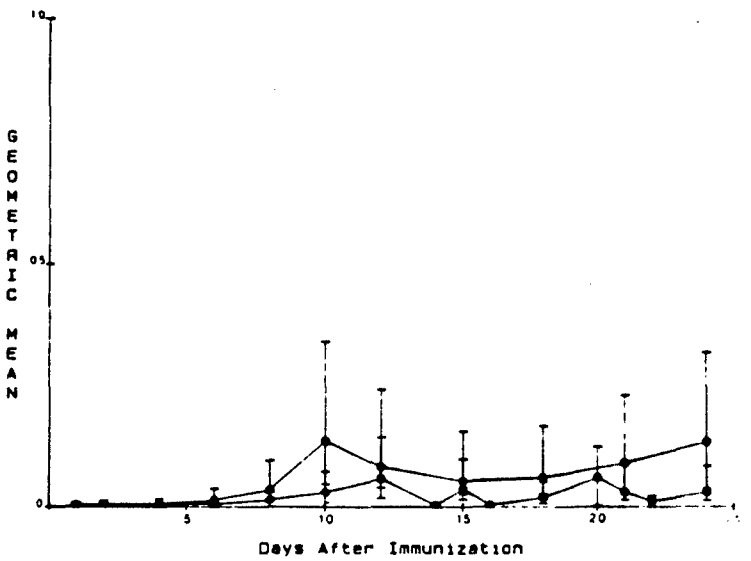

Figure 3. Geometric mean IgG anti-shigella LPS responses in isolated ileal loop secretions from the two groups of rabbits from figure 2 are shown.

C. Mucosal response following combined parenteral immunization with heat-killed Shigella X16 and oral immunization with live Shigella X16. The overall kinetics of the immune response achieved in these group III animals (Table I) followed those of the primary mucosal immune response. However, the mean IgA anti-Shigella LPS activity was greater for all days in the groups III animals. In addition, the IgA anti-Shigella LPS activity was significantly stronger on day 10 in Group III (Figure 2). In these animals, there was a significant amount of IgG anti-Shigella LPS activity present in the intestinal secretions. The response for IgG was more variable than that of secretory IgA, however, the kinetics parallel those of the primary secretory IgA response. By day 6 after oral immunization, the IgG anti-Shigella in secretions had increased significantly over day 0 values (Figure 3). These findings indicate that an augmentation of the primary immune response in intestinal secretions can be achieved by using a combination of parenteral immunization with oral immunization. The timing of the doses must, however, be carefully orchestrated.

D. Immune responses following parenteral stimulation with heat-killed Shigella X16 in complete Freund's adjuvant (CFA) and oral immunization with live Shigella X16. The group IV rabbits were used to determine whether CFA given with the heat-killed antigen would enhance or prolong the secretory IgA response to Shigella X16 using the combined parenteral and oral immunization schedule (Table I). There was no significance increase in the IgA anti-Shigella LPS response of these animals as compared to the group III animals. As with the group III animals, a significantly stronger local response was seen in these animals as compared to the group II animals (Figure 4). A control group given only parenteral heat-killed Shigella in CFA (group V) gave a weak variable response which lagged behind the primary IgA response seen from a single oral dose of live Shigella (Figure 4). Only weak IgG anti-Shigella LPS activity was found in secretions from the group IV animals and almost no such activity was seen with the group V rabbits (Figure 5), despite the fact that the latter animals had high serum IgG activity to Shigella (Tables III and IV). Interestingly, we detected a 50% decline in the serum IgG anti-Shigella LPS activity in serum from animals receiving combined parenteral antigen in CFA and oral antigen (group IV) versus parenteral antigen in CFA alone (group V). This type of decline may reflect the production of a suppressor T cell for the IgG response by the oral priming. This type of mechanism has been described by several workers in other animal systems (18).

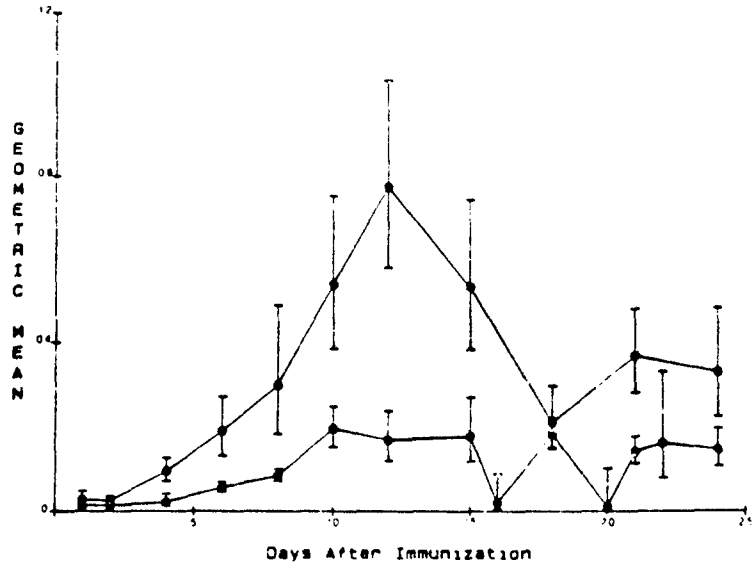

Figure 4. Geometric mean IgA anti-shigella LPS responses in isolated ileal loop secretions from rabbits given a single parenteral dose of killed shigella in CFA (closed circles) and from rabbits given a combined parenteral dose of antigen in CFA on day -1 with the single oral dose of live antigen on day 0 (open circles) are shown. SEM are indicated.

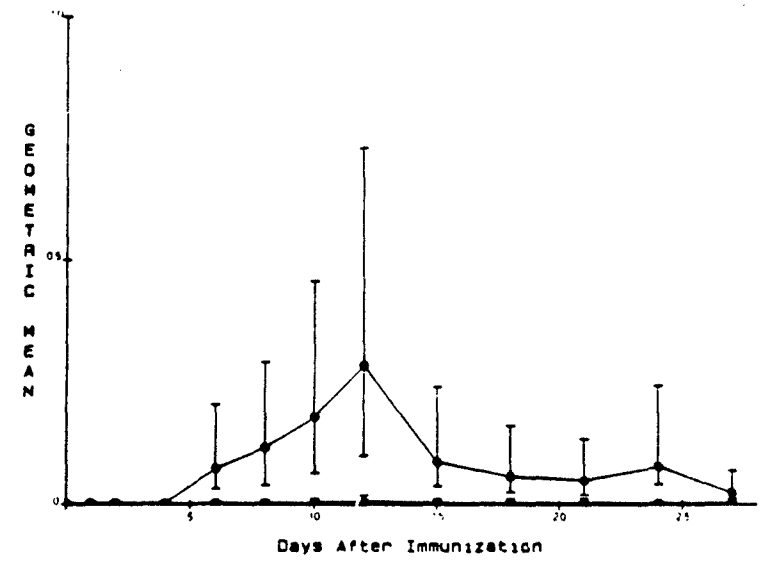

Figure 5. Geometric mean IgG anti-shigella LPS responses in isolated ileal loop secretions from the two groups of rabbits from figure 4 are shown.

Table III. Serum IgG and IgA Anti-Shigella LPS Activity in Rabbits Immunized with Antigen via both Intramuscular with CFA and Oral Routes.

Days Postimmunization[1]	IgA Activity[2]	IgG Activity[2]
Preimmunization	.028 (.018-.045)	.018 (.010-.033)
6-7	.485 (.453-.518)	.482 (.445-.522)
8-14	.743 (.669-.826)	.803 (.762-.846)
15-21	.784 (.660-.932)	.823 (.817-.933)
22-28	.819 (.721-.932)	.930 (.862-1.003)
29-35	.715 (.583-.878)	.924 (.831-1.027)
36-42	.889 (.794-.996)	.987 (.930-1.029)

1. Day of intramuscular immunization with Heat-killed shigella in CFA = day -1. Oral dose of live shigella given on day 0.
2. Results expessed as Geometric mean with the variance as described in the Methods section.

Table IV. Serum IgG and IgA Activity to Shigella LPS in Rabbits Given Heat-Killed Shigella in CFA Intramuscularly

Days Postimmunization	IgA Activity[1]	IgG Acitivity[1]
preimmunization	.018 (.011-.028)	.015 (.007-.032)
6-7	.308 (.180-.525)	.243 (.120-.493)
8-14	.670 (.494-.908)	1.294 (1.050-1.596)
15-21	.380 (.494-.908)	1.096 (.889-1.352)
22-28	.540 (.450-.647)	1.941 (1.607-2.344)
29-35	.273 (.166-.448)	1.728 (1.380-2.168)
36-42	.565 (.469-.681)	1.690 (1.285-2.223)

1. Activity is expressed as Geometric mean with the variance as described in the Methods section.

II. Mucosal Immune Response to Shiga Toxin.

In Figure 6 is shown the data from intestinal secretions of 2 rabbits given direct intraloop immunization with Shiga toxin as described in the methods section. We found that by day 10, a significant increase in Shiga toxin over background was seen in both rabbits. Booster doses of Shiga toxin were given on days 7 and 14. The antibody activity continued to rise through the end experiment at one month. Secretions from these animals were sent to Dr. Ed Brown for analysis of the ability of the IgA anti-Shiga toxin to prevent the in vitro pathogenic effects of this molecule. As shown on the Y2 axis (inhibition titer $\log_2$) there was an excellent correlation of the IgA anti-Shiga toxin activity with the inhibition titer determined by Dr. Brown's assay. These findings indicate that there is a great potential for secretory IgA to prevent the toxic activity of Shiga toxin. Future studies will be directed to confirming these results in a larger group of animals and to establishing whether a secretory IgA memory response against Shiga toxin can be elicited.

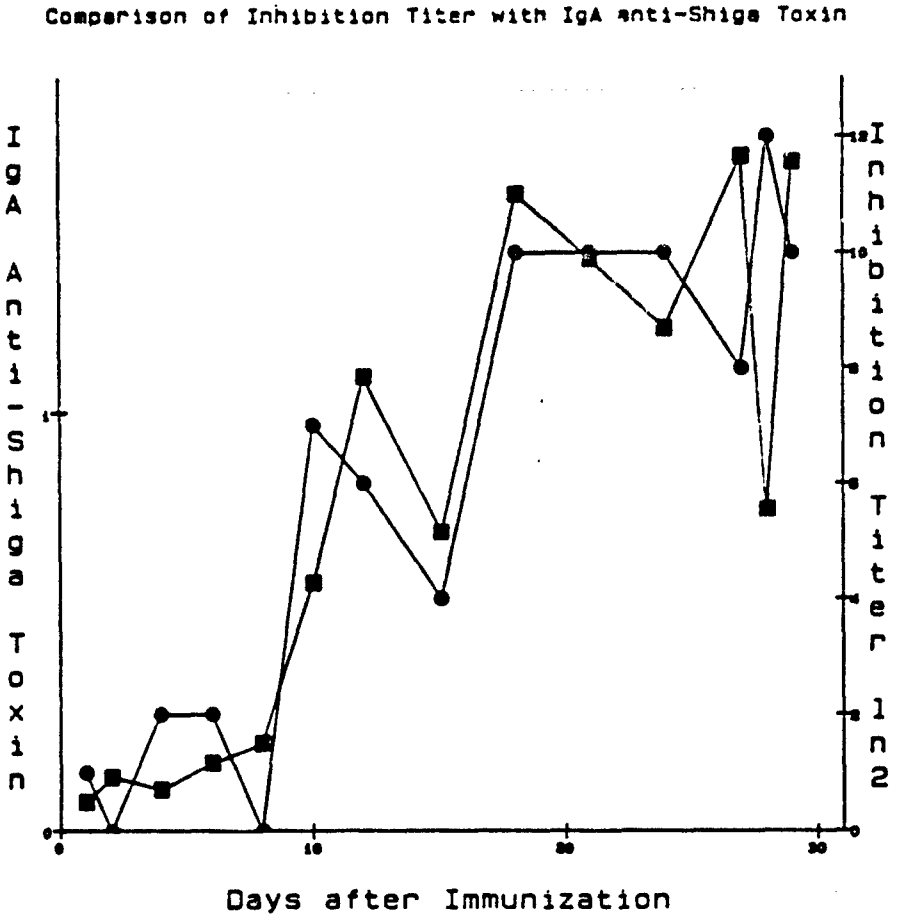

Figure 6. Mean IgA anti-Shiga toxin activity (ELISA) (squares) correlates well with the mean inhibition titer (circles) in intestinal secretions from immunized rabbts (see text).

III. Correlation of the IgA response in intestinal secretions with cellular events.

Last year, we established cell culture techniques for following mucosal stimulation by enteropathogens. In the past year, we have begun to correlate the IgA response in intestinal secretions with cellular events. In the present studies, rabbits were given the combined parenteral and oral immunizations schedule described above in group III animals. To determine the development of the cellular response, mononuclear cell preparations from Peyer's patches, appendix, spleen, mesenteric and axillary lymph nodes were studied. Spleen mononuclear cells from day 8 rabbits were used as they had the most reliable and largest amount of anti-Shigella activity. As can be seen in Figures 7 and 8, the optimum cell concentration range was from 2-8 x 10^6 viable cells per ml for both IgG and IgA anti-Shigella activity. Increasing time of incubation did not raise the amount of anti-Shigella activity at suboptimal cell concentration to those seen with optimal concentrations. Maximal anti-Shigella concentrations were usually reached between 4 and 14 days of incubation.

These findings indicate that the parenteral priming together with the oral immunization is able to stimulate both systemic IgG and IgA anti-Shigella B cells in several lymphoid tissues. Further studies will determine whether the IgA response is enhanced in the memory studies. This will provide a key basis for following cellular events after use of oral vaccination.

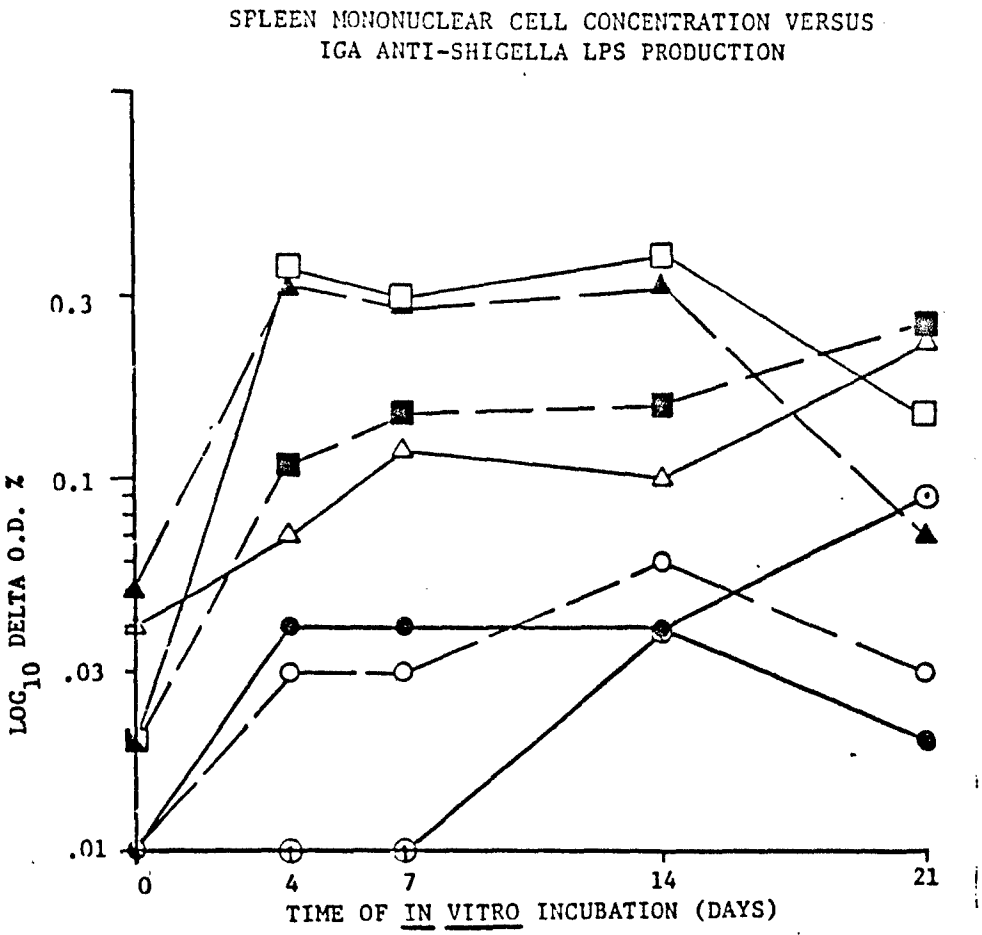

Figure 7. IgA anti-Shigella LPS production. Spleen mononuclear cell concentrations (x 10^6 /ml) are: -0.5 (circle with a dot); 1.0(filled circle); 2.0(open circle); 4.0(filled triangle); 8.0(open triangle); 16.0(open square); and 32.0(filled square). The IgG anti-Shigella LPS activity was approximately 10 fold greater than that for IgA as shown in Figure 2.

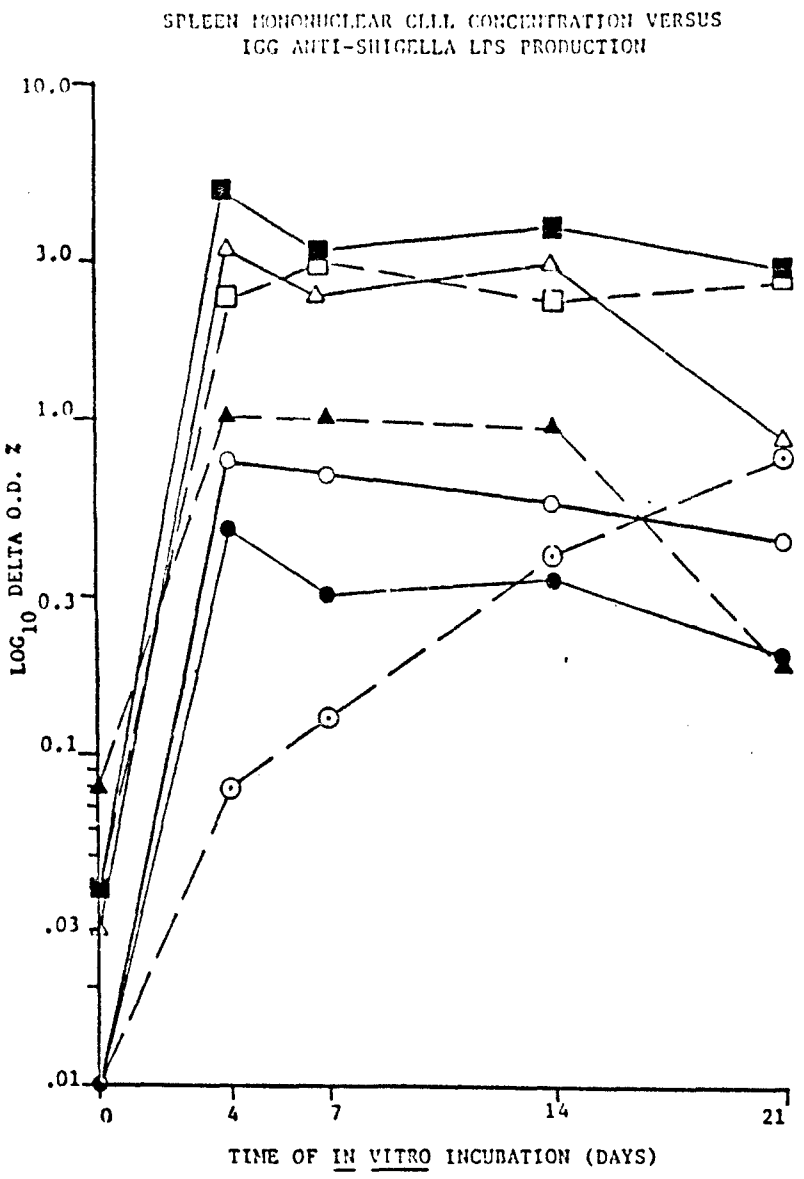

Figure 8. IgG anti-Shigella LPS production. Spleen mononuclear cell concentrations (x 10^6 /ml) are: -0.5 (circle with a dot); 1.0(filled circle); 2.0(open circle); 4.0(filled triangle); 8.0(open triangle); 16.0(open square); and 32.0(filled square).

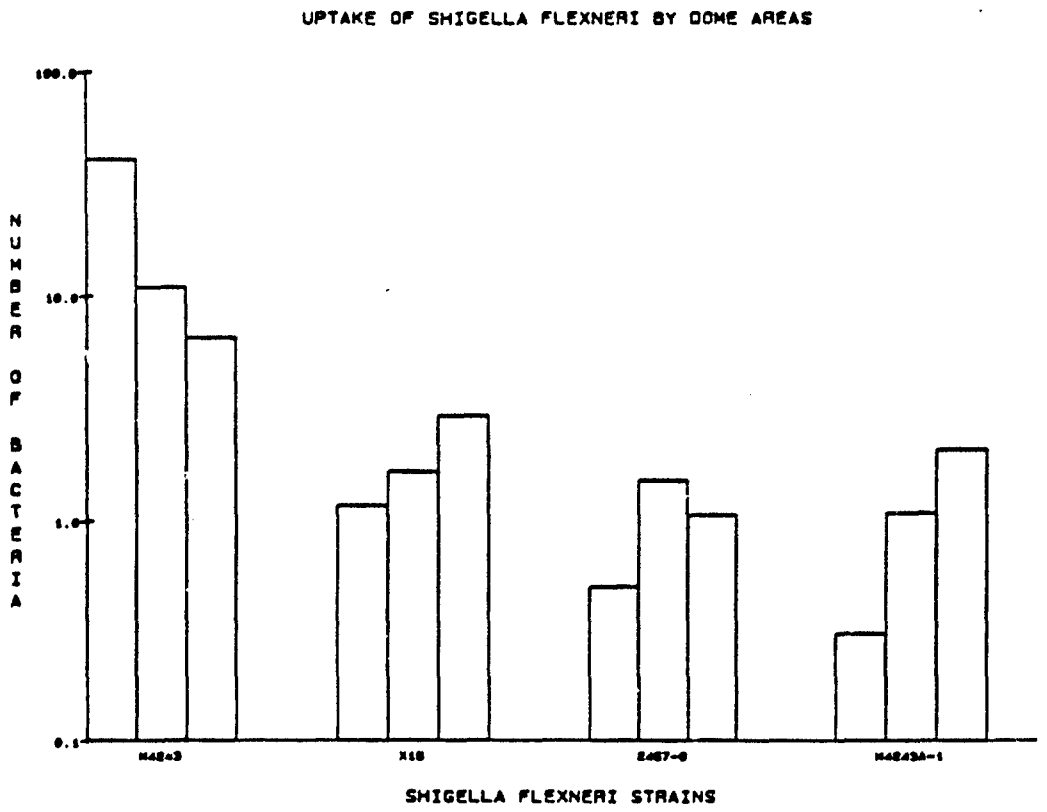

Figure 9. Uptake of shigella by epithelium overlying dome areas of Peyer's patches correlate with the inherent pathogenicity of the strain. Each bar represents the average number of bacteria per dome area in a rabbit. Invasive M4243, had the most uptake. However, the strongest IgA anti-Shigella LPS was elicited by the least pathogenic strains 2457-0 and $M4243A_1$ (6).

IV. Shigella uptake studies in vivo.

Our laboratory has made two major observations with regard to Shigella flexneri. First, to develop a significant mucosal memory response, one need not use invasive Shigella. This implies that the mucosal immune system is able to take viable Shigella up from the intestinal lumen even though they lack the virulence plasmid. The second major observation we have made relates to the pathogenicity of Shigella. We have noted that the virulent strains of Shigella prefer invading and creating ulcerations in areas where there are Peyer's patches. This implies that the initial uptake of the Shigella may be influenced by the presence of M cells in the follicle-associated epithelium. To determine the relationship between the initial uptake of Shigella and the immune response to Shigella, we have performed a series of experiments examining strains with different invasive capabilities. As shown in Figure 9, after 90 minutes within the intestinal lumen, there is a marked difference between the uptake of strain M4243 (virulent containing the 140 megadalton plasmid) and the less virulent strains. The uptake by the dome area correlates well with the inherent invasive capabilities of the individual strains.

We are now reviewing the invasiveness of these strains over the villi. There was no correlation between invasiveness of these strains and the ability to elicit a mucosal memory response. The findings indicate that the uptake of Shigella by M cells over the dome areas may serve as the portal of entry for this enteropathogen as well as for immune processing of these cells. A similar route for invasion by HIV was suggested recently by Sneller and Strober (13). Once within the M cell, pathogenic strains may be capable of replicating while nonpathogenic strains do not. Future work will be directed to determining whether secretory IgA can prevent uptake of these strains.

V. Response of Paneth Cells to Intestinal Flora.

In our isolated ileal loop model, we have repeatedly observed a striking hypertrophy and hyperplasia of Paneth cells which occurs as the loops age. This accompanied by an atrophy of villi with an increase in crypt depth (1). Whereas the known trophic effects of chyme, lost to the isolated loops, have been suggested as an explanation for the changes seen, isolation was not the only variable characterizing the model. We have shown that within days of their creation, there is extensive bacteria columnization of these isolated ileal loops (3). In the present studies, we have found that by controlling the bacterial overgrowth with antibiotics, we can prevent the Paneth cell changes. For these studies, antibiotics were instilled directly into 1 of 2 isolated ileal loops created in the same rabbit. Nonabsorbable antibiotics (see method) were used. Therefore, results for these animals include an antibiotic loop (AL) a saline loop (SL); and the control ileum (CI). As shown in Table V there was a significant there was a significant reduction in he Paneth cell hyperplasia and in the crypt depth in the antibiotic loop as apposed to the saline loop. No differences were found in the

numbers of mitoses in either loop or the control ileum. In both the antibiotic and saline loop, there was an increase in number of goblet cells compared to the control ileum. These findings indicate that the Paneth cell hyperplasia is largely in response to the microbial flora of the gut. As such, it suggests a role for the Paneth cell in responding to microorganisms in the gut lumen. Future studies will center around purifying Paneth cells from our hyperplastic loops and evaluating their ability to both phagocytose and destroy microorganisms which have been variously coated with IgA or IgG anti-Shigella.

Table V. Paneth Cells and Crypt Epithelium Respond to Microorganism in Isolated Ileal Loops.

Cond	Villus Height (um)	Crypt Depth (um)	Paneth Cells (um)	Mitoses (x/10 HPF)	Goblet Cells (x/EN)	IEL (x/EN)	Flora Week 1 (grade)	Flora Week 2 (grade)
AL	286±21*	99±6	55±7	26±4	.120±.022	.511±.045	1.6±.1	1.5±.1
SL	301±14	139±9	79±7	22±3	.137±.022	.492±.057	3.5±.2	3.9±.1
CI	441±31	90±4	24±2	23±5	.051±.008	.341±.029		

* Data, Mean ± SEM

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